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IMMUNE RESPONSE TO *PLASMODIUM BERGHEI* SPOROZOITE ANTIGENS. I. EVALUATION OF MURINE T CELL REPERTOIRE FOLLOWING IMMUNIZATION WITH IRRADIATED SPOROZOITES

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Abstract. The *Plasmodium berghei* sporozoite antigen-specific T cell repertoire was analyzed in C57BL/6 (H-2^b), BALB/c (H-2^d) and C3H/HeN (H-2^k) mice following immunization with irradiated sporozoites. Proliferative responses were correlated with the protective status of each strain. Proliferative reactivities to sporozoite antigens were compared in cultures containing either $CD4^+$ T cells, $CD8^+$ T cells, or total splenic lymphocytes. $CD8^+$ T cells had no proliferative activity to sporozoite antigens; $CD4^+$ T cells and splenic lymphocytes responded to the priming antigen, but the responses varied according to the mouse strain tested. The proliferative activity diminished at the onset of protection, presumably due to the induction of regulatory or non-proliferative T cell subsets. Sporozoite-immune lymphocytes did not respond to *P. berghei* circumsporozoite synthetic peptides. The restricted utilization of T cell epitopes during anti-sporozoite responses can be interpreted as resulting in part from a limited processing of the CS protein antigen.

Information regarding T cell responses to complex antigens found on parasites, like the protozoan *Plasmodia*, is incomplete and fragmentary. Throughout this study, we were guided by the lessons learned from extensively studied immune responses to soluble protein antigens. The usefulness of this approach should be critically evaluated so that studies dealing with immune reactivities elicited by antigenically complex parasites could either assume new directions or continue to rely on the models provided by the well-characterized protein antigen systems.

The current understanding of the role of T lymphocytes in immunity stems from nearly 2 decades of research investigating immune responses directed against well-characterized soluble protein antigens, including hen egg lysozyme,[1] myoglobin,[2] cytochrome C,[3] β-galactosidase,[4] and viral antigens such as influenza hemagglutinin.[5] T cells show exquisite antigen specificity, considered to be the property of the clonally expressed T cell receptor (TCR) that is formed by the rearrangement of the TCR gene families.[6] Fine antigen specificity is further controlled by the gene products of the major histocompatibility complex (MHC) at the level of T cell repertoire selection and antigen-specific T cell recognition and activation.[7] The presence of cell surface markers, such as CD4, CD8, and Thy 1, on T lymphocytes has facilitated dissociation of functional T cell specificities,[8, 9] allowing each sub-population to be studied separately. $CD4^+$ T cells generally function as inducer cells, collaborating with B cells in antibody production, while the $CD8^+$ T cells function either as cytolytic cells (eliminating virus-infected target cells) or as suppressor T cells (regulating immune functions).

The realization that T cells are activated by metabolically degraded antigenic fragments has validated the use of synthetic peptides or enzymatically and chemically cleaved fragments from native antigens as probes to map T cell epitopes.[10–13] Although extensive T cell epitope mappings have been done in other well-characterized protein systems,[1–4] there has been limited characterization of T cell responses induced by protozoan parasites, for example, the rodent malaria *Plasmodium berghei*.[14]

Protection against the infective sporozoite stage of *P. berghei* can be induced by prior immunization with irradiated sporozoites.[15, 16] Studies have shown that the circumsporozoite (CS) protein contains immunodominant B cell determinants localized within the central repeat region of the CS protein that stimulate IgG response.[17, 18] Immune protection is mediated in part by CS antibodies, since CS monoclonal antibodies (Mabs) can transfer protection.[19, 20] The involvement of $CD8^+$ T cells in protection has

also been demonstrated in vivo by the depletion of selected T cell subpopulations.[21,22] In elegant experiments by Romero and others, sporozoite-derived cytolytic T cell clones specific for aa 249–260 have been demonstrated to induce protection following adoptive transfer into naive mice.[23] Despite these observations, the role of CS protein-specific T cells in protection against malaria is incompletely understood. The identification of the CS protein immunodominant T cell epitopes activating T cells either for a helpful or a regulatory immune response during sporozoite protection is essential for a better understanding of anti-sporozoite immunity and vaccine development. We examined the CS protein-specific T cell repertoire induced by irradiated sporozoite antigens in several murine strains capable of generating protective immunity against live sporozoite challenge.

MATERIALS AND METHODS

Mice

Female BALB/c (H-2^d), C57BL/6 (H-2^b), and male C3H/HeN (H-2^k) mice were obtained from Jackson Laboratories (Bar Harbor, ME) and used at 6–12 weeks of age.

Sporozoite preparation

Sporozoites dissected from the salivary glands of infected *Anopheles stephensi* mosquitoes were provided by Imogene Schneider, Department of Entomology, Walter Reed Army Institute of Research, Washington, DC. Upon dissection, sporozoites were placed in medium 199 (Gibco, Grand Island, NY) containing 3% normal mouse serum (NMS). For immunizations, portions of sporozoites were irradiated at 15,000 rads from a cobalt source and stored on ice until the time of immunization. Crude sporozoite preparations used as antigen to stimulate lymphocyte cultures were irradiated at 30,000 rads and then stored at −70°C.

Mosquito salivary gland antigen

Non-infected mosquito salivary glands were sham dissected and the debris was treated in an identical manner to sporozoites. Gland debris was used at concentrations equivalent to 10^4 sporozoites/gland.

Immunizations

To induce sporozoite-reactive splenic lymphocytes, all mouse strains were immunized iv with 75,000 irradiated sporozoites followed by 2 weekly boosters of 20,000 irradiated sporozoites iv. Representative mice were challenged iv with 10,000 live sporozoites. Sporozoite-specific lymph node cells were induced by priming peripherally with 0.1 ml of 50,000 irradiated sporozoites emulsified 1:1 (v/v) with Complete Freund's Adjuvant (CFA; Difco, Detroit, MI). Control animals received an equal volume of phosphate buffered saline in CFA (PBS-CFA).

Parasitemia screening and protection

Individual thin blood smears were taken daily starting 5 days after challenge and stained with Giemsa. One hundred oil immersion fields were viewed for parasitized erythrocytes. Mice were considered protected if parasites were not present after 14 days.

Peptides

P. berghei CS protein synthetic peptides were purchased from Peninsula Laboratories (Palo Alto, CA). Amino acid sequences are as follows:
(61–75), RNTVNRLLADAPEGK;
(69–87), ADAPEGKNEKKNEKIERNN;
(81–94), EKIERNNKLKQPPN;
(86–94), NNKLKQPPN;
REPEAT, DPAPPNANDPAPPNANDPAPPNAN;
(245–260), SYIPSAEKILEFVKQ;
(245–270), SYIPSAEKILEFVKQIRDSITEEWSQ;
(256–270), FVKQIRDSITEEWSQ;
(261–283), RDSITEEWSQCNCVGSGIEVRKR;
(280–297), VRKRGSNKKAELTLRDID;
(298–314), TEICKMKMCSSIENIVS.

Media

RPMI 1640/EHAA medium (Microbiological Associates, Walkersville, MD) enriched with 100 μ/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), 4 μM glutamine (Gibco), 2.5 μM sodium pyruvate (Gibco), 10 μM non-essential amino acids (Gibco), 50 mM 2-mercaptoethanol (Bio-rad, Richmond, CA), and 0.5% NMS was used.

Splenic lymphocyte population

Sporozoite-immune mice were killed by cervical dislocation 7 days after the last boosting dose of sporozoites. Their spleens were dissected aseptically and placed in Hank's Balanced Salt Solution (HBSS) (Gibco). After 3 cell washes, the final cell concentrations were adjusted to 5×10^6 cells/ml of complete medium for culture.

Lymph node cell population

Sporozoite-CFA and PBS-CFA primed lymph node cells were obtained from mice 10–12 days after priming and prepared for culture as described for spleen cells.

$CD4^+$ and $CD8^+$ T cell depletions

Splenic cell populations were depleted of $CD4^+$ or $CD8^+$ T cells using culture supernatants of the hybridoma 2RL specific for $CD4^+$ or hybridoma 83-12-5 specific for $CD8^+$ (both gifts from Richard Hodes, National Institutes of Health, Bethesda, MD). Cells (20×10^6/ml) were incubated with a 1:3 dilution of supernatant in HBSS at 4°C for 1 hr. Cells were pelleted and resuspended in Low Tox Rabbit Complement (Cedar Lane Laboratories, Westbury, NY) at a 1:10 final dilution in HBSS containing 1% fetal bovine serum (FBS; Gibco) and incubated at 37°C for 1 hr. Following incubation, the cells were centrifuged at $400 \times g$ for 10 min and washed twice in HBSS. $CD4^+$ and $CD8^+$ enriched populations were resuspended to 5×10^6 cells/ml of complete medium.

Cell preparation for cytofluorometric analysis

Whole spleen and selectively depleted populations were labeled with Mabs against cell surface markers. Briefly, 10^6 cells from each group were washed in PBS containing 0.5% FBS and 0.1% sodium azide. Cells were resuspended in PBS/FCS/sodium azide buffer and incubated with 4 μl fluorescein-conjugated goat anti-mouse-CD4 or -CD8 (Becton Dickinson, Mt. View, CA) at 4°C for 1 hr. Cells were pelleted by centrifugation at $400 \times g$ and washed twice in PBS/FBS/sodium azide. Labeled cells were fixed in 1% formaldehyde in a final volume of 1 ml and stored in the dark at 4°C until analyzed on a Fluorescence Activated Cell flow cytometer (FACscan, Becton Dickinson).

Proliferative assay

Unfractionated and $CD4^+$ and $CD8^+$ enriched T cell populations and unfractionated lymph node cells were cultured in 96-well microtiter plates (Costar) at a final concentration of 5×10^5 cells/0.2 ml of complete medium containing sporozoite antigens from 300 to 3,000 frozen/thawed/30,000 rads irradiated sporozoites, or equivalent amount of mosquito gland debris, or synthetic peptides at concentrations ranging from 0.01 to 10 μg/ml. Cells were cultured for 4 days at 37°C in a humidified atmosphere containing 5% CO_2. During the last 16 hr of culture, 1 μCi of tritiated thymidine (^{3}H-TdR; New England Nuclear, Boston, MA) was added to each well to quantitate proliferative activity. Cultures were harvested using an automated sample harvester (Skatron, Sterling, VA) and analyzed by scintillation spectroscopy.

RESULTS

Sporozoite-primed spleen cells proliferate to sporozoite antigen in culture

To characterize sporozoite antigen-specific responses, sporozoite-immune spleen cells from BALB/c (H-2^d), C57BL/6 (H-2^b), and C3H/HeN (H-2^k) mice were cultured with irradiated, frozen/thawed sporozoite antigens. This particular immunization regimen was chosen for analysis because previous studies showed that it usually induces immune protection against challenge with 10,000 live sporozoites.[15, 16] BALB/c mice demonstrated a strong anti-sporozoite proliferative response, whereas the C57BL/6 strain demonstrated only a moderate proliferative reactivity and C3H/HeN usually did not respond to sporozoite antigens (Fig. 1A). The variability in the proliferative responses of splenic lymphocytes suggests strain-dependent sensitivity to sporozoite antigens. However, comparison of proliferative responses of sporozoite-primed lymph node cells obtained from BALB/c, C57BL/6, and C3H/HeN mice demonstrates the ability of all 3 strains to respond to sporozoite antigens in culture (Fig. 2). Therefore, strain-associated variations in proliferative reactivities may be unique to splenic cultures and may be caused by iv im-

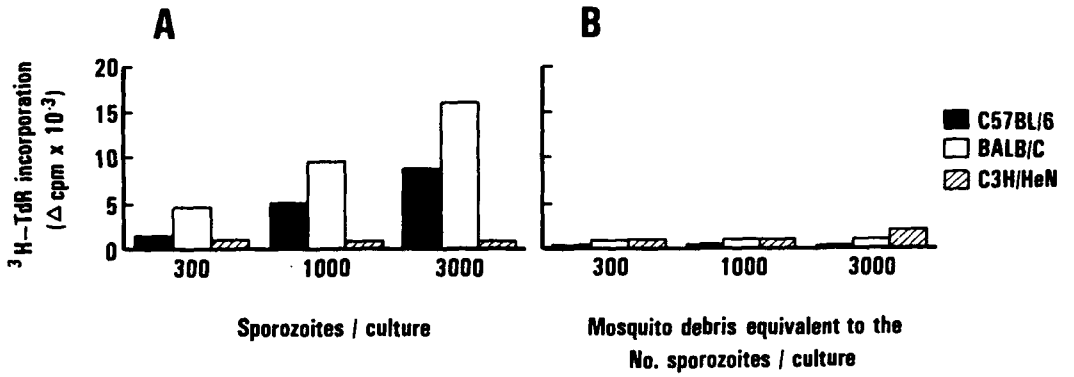

FIGURE 1. Proliferative reactivity of sporozoite-induced spleen cells. BALB/c (H-2^d), C57BL/6 (H-2^b), and C3H/HeN (H-2^k) mice were primed iv with 75,000 irradiated sporozoites and boosted twice with 20,000 irradiated sporozoites 1 week apart. Seven days later, pooled spleen cells from 5 mice/group were cultured with (**A**) graded amounts of sporozoite antigen (equivalent to 300, 1,000, or 3,000 sporozoites) or (**B**) graded amounts of mosquito salivary gland debris equivalent to the number of salivary glands yielding comparable numbers of sporozoites. Control cultures contained medium alone. Data represent mean responses of triplicate cultures in cpm of ^{3}H-TdR incorporation during the last 16 hr of culture.

munization with irradiated sporozoites without CFA.

To ascertain the specificity of anti-sporozoite antigen responses, graded amounts of mosquito salivary gland debris obtained from a sham sporozoite dissection of uninfected mosquitoes were added to sporozoite-primed splenic lymphocytes and the degree of proliferation was assessed. As shown in Figure 1B, the control antigen induced only a baseline level of proliferative activity, verifying antigen specificity of the responses. CFA-primed lymph node cells from BALB/c, C3H/HeN, and C57BL/6 mice did not respond to sporozoite antigens in vitro, but responded to purified protein derivative (data not shown). This demonstrated that the irradiated sporozoites were not mitogenic (Fig. 2).

Dissociation of protection and proliferative T cell activity

To establish the minimum number of immunizations required for induction of both a proliferative response and protection, BALB/c, C57BL/6, and C3H/HeN mice were given either a single dose of 75,000 irradiated sporozoites, a priming dose of 75,000 irradiated sporozoites followed by a booster of 20,000 irradiated spo-

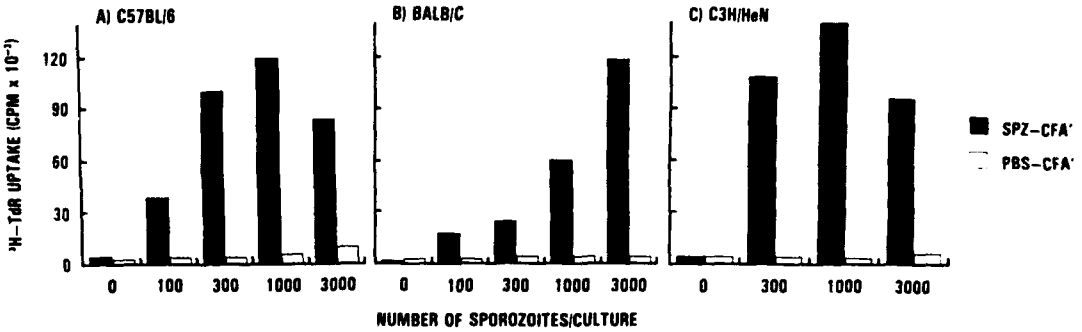

FIGURE 2. Proliferative reactivity of sporozoite-CFA-primed lymph node cells (LNC). C57BL/6, BALB/c, and C3H/HeN mice were primed with 50,000 sporozoites with CFA (SPZ-CFA) or PBS-CFA; 14 days later, a pool of draining LNC from 3 mice/group were cultured with graded amounts of sporozoite antigen (equivalent to 100–1000 SPZ per culture), mosquito salivary glands (yielding comparable numbers of sporozoites), or medium alone. Antigen-specific proliferative activity is represented as mean responses of triplicate cultures in cpm of ^{3}H-TdR incorporation during the last 16 hr of culture. Responses of PBS-CFA primed mice to 2.5 μg/ml purified protein derivative in culture ranged from 150,000 to 200,000 cpm.

rozoites, or a priming dose of 75,000 irradiated sporozoites followed by 2 boosters of 20,000 irradiated sporozoites each. Pooled splenic lymphocytes from each group (5 mice/group) were assayed in vitro for sporozoite-specific proliferative T cell responses. Additionally, groups of 5 mice from each strain, representing each of the 3 immunization strategies, were challenged iv with 10,000 live sporozoites and subsequently screened for parasitemia.

Sporozoite antigens-specific T cell responses were consistently observed in BALB/c and C57BL/6 strains, although the magnitude of reactivities differed in each strain and was affected by the immunization regimen (Fig. 3A–F). BALB/c spleen cells showed extremely low levels of sporozoite antigen-specific proliferative activity (3-fold increase over the background control) after immunization with 75,000 irradiated sporozoites, a dose that fully protects this strain against sporozoite challenge (Fig. 3D, Table 1). However, splenic lymphocyte cultures obtained from BALB/c mice after priming with the latter 2 protocols showed enhanced proliferative responses with a 10-fold increase over the background controls (Fig. 3E,F). In contrast, sporozoite-primed spleen cells from C57BL/6 mice proliferated well to sporozoite antigens in culture (6-fold increase over the background control), but the mice were not fully protected until after 3 doses of sporozoites. By that time, the proliferative response had diminished to low levels (3-fold increase over the background control; Fig. 3A–C, Table 1). Generally, proliferation of sporozoite-primed C3H/HeN splenic lymphocytes was not observed, but protection was induced in most mice in 3 out of 4 experiments although it required the full 3 dose immunization schedule (Fig. 3G–I, Table 1). With a single exception (Exp. 2, Table 2), proliferative activity was evident in the splenic lymphocytes and those animals were also protected.

$CD4^+$ T cells are the proliferating population to sporozoite antigens in culture

To determine which T cell sub-populations responded to sporozoite antigen(s) in culture, mice from each strain were immunized according to the 3 separate immunization schedules. Seven days after the last immunization, splenic lymphocytes were negatively selected for $CD4^+$ T cell and $CD8^+$ T cell populations. The purity of the enriched T cell populations was analyzed by cytofluorometry using fluorescein derivatized Mabs specific for CD4 and CD8 markers. The results showed enrichment of the selected population with 0.7–5% of the depleted T cells remaining (data not shown).

As with the unfractionated spleen cells, the $CD4^+$ and $CD8^+$ T cell sub-populations derived from BALB/c mice primed with 75,000 sporozoites failed to proliferate to sporozoite antigens in culture (Fig. 3D). Sporozoite-specific proliferation was observed, however, in cultures of unfractionated and $CD4^+$ T cells immunized with 75,000 irradiated sporozoites followed by 1–2 boosters with 20,000 irradiated sporozoites (Fig. 3E, F). $CD8^+$ T cells never proliferated in response to sporozoite antigens and their removal did not significantly enhance the response of $CD4^+$ T cells. Likewise, $CD4^+$ T cells, but not $CD8^+$ T cells, obtained from sporozoite-immune C57BL/6 spleens proliferated in response to sporozoite antigens in culture. Although the proliferative reactivity of the $CD4^+$ T cells paralleled that of the unfractionated spleen lymphocytes, their reactivity was generally lower. The reasons for this reduction are unclear, although it is possible that some proliferative populations might have inadvertently been eliminated during the negative selection process. Alternatively, within the unfractionated spleen, the proliferating $CD4^+$ T cell population produces IL-2, a lymphokine necessary for proliferation of the $CD8^+$ T cells. Therefore, in the unseparated population, $CD8^+$ T cells will also proliferate, which may contribute to the enhanced proliferation of the unfractionated splenic population compared to the $CD4^+$ T cells alone. Upon removal of the $CD4^+$ T cells, IL-2 production is eliminated and $CD8^+$ proliferation does not occur. Experiments in which IL-2 was added to the $CD8^+$ T cell population showed an overall increase in the proliferative responses, and therefore sporozoite antigen-specific responses were difficult to elucidate (data not shown).

Since unfractionated C3H/HeN splenic lymphocytes seldom generated sporozoite-specific proliferative responses, proliferation of the $CD4^+$ T cell set after depletion of the $CD8^+$ T cell population was not expected (Fig. 3H, I). The unmasking of sporozoite-reactive $CD4^+$ T cells became evident in splenic cultures obtained from mice immunized with 75,000 irradiated sporozoites followed by 1–2 boosters of 20,000 irra-

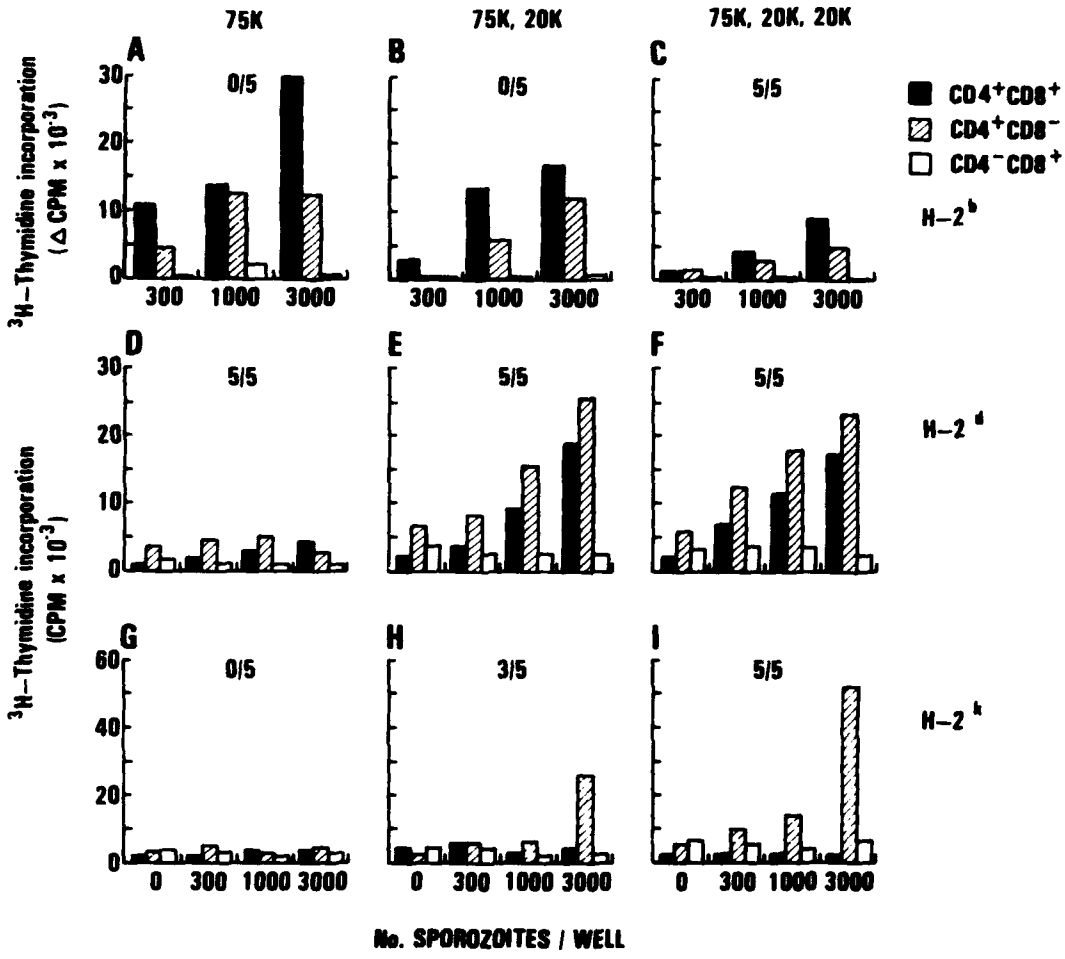

FIGURE 3. Proliferative reactivity of splenic T cell sub-populations to sporozoite antigens. Unseparated spleen cells, enriched CD4+ T cells and CD8+ T cells obtained from 5 mice/group were cultured in the presence of graded amounts of sporozoite antigen equivalent to 300, 1,000, or 3,000 sporozoites. Data represent mean responses of triplicate cultures as cpm (panels D–I) or Δcpm (panels A–C) of ^{3}H-TdR incorporation during the last 16 hr of culture. Representative mice from each immunization schedule were subsequently challenged with 10,000 live sporozoites iv and screened for parasitemia 5–14 days after challenge. The number of protected mice vs. the total number of mice challenged/group is indicated following the immunization regimen. Control animals challenged with 10,000 live sporozoites displayed parasitemia by day 7 post-challenge.

diated sporozoites. Although in all 3 strains the CD4+ T cells proliferated to sporozoite antigens, it was evident that at least in the C3H/HeN strain the induction of this T cell type might be directly controlled by regulatory CD8+ T cells. Thus, sporozoite priming may activate functionally distinct T cell subsets in different mouse strains.

Data presented in Table 2 also shows that the immune protection in C3H/HeN (H-2^k) mice seems to be accompanied by the expansion of CD4+ T cells. In Exp. 3 (Table 2), when the CD4+ T cell population did not proliferate in response to sporozoite antigens, the mice were not protected. However, data obtained from the A/J mouse strain demonstrated a lack of anti-sporozoite antigen proliferation in both T cell subpopulations, yet the mice were solidly protected (Table 2).

Sporozoite-specific T cells do not recognize CS protein peptides in culture

Having established that sporozoite antigen-specific CD4+ T cells are inducible following im-

TABLE 1

Immunization with irradiated sporozoites induces protection against live sporozoite challenge

Strain	Immunization	Challenge	Percent protected	No. mice	Anti-SPZ proliferative T cell responses*	
					Spleens	CD4+
BALB/c	0	10,000	0	15	−	−
	75,000	10,000	100	10	−	−
	75,000; 20,000	10,000	100	10	+	+
	75,000; 20,000; 20,000	10,000	100	15	++	++
C57BL/6	0	10,000	0	15	−	−
	75,000	10,000	0	10	+++	++
	75,000; 20,000	10,000	40	10	++	++
	75,000; 20,000; 20,000	10,000	100	15	+	+
C3H/HeN	0	10,000	0	15	−	−
	75,000	10,000	0	15	−	−
	75,000; 20,000	10,000	60	15	−	+
	75,000; 20,000; 20,000	10,000	100	15	−	++

Mice were screened for erythrocytic parasites 5–14 days post-challenge. Data were collected from 3 separate experiments involving 18 separate sporozoite dissections.

* Anti-sporozoite proliferative responses are as follows: no stimulation (−), 2-fold stimulation (+), 3–5-fold stimulation (++), or >5-fold stimulation (+++) over medium controls. Stimulation was calculated as cpm from cultures stimulated with antigens over the cpm from cultures with medium alone.

munization, we next analyzed the fine specificity of these T cell responses. For this purpose, synthetic peptides representing part of the repeat region and the flanking regions of the CS protein were used to probe for T cells with anti-CS peptide reactivity (Fig. 4). None of the peptides were able to recall antigen-specific responses of splenic lymphocytes, $CD4^+$- or $CD8^+$-enriched T cells from all 3 strains of mice regardless of the immunization protocol or the peptide concentrations tested (0.01–10 μg/ml). Representative data obtained with 10 μg/ml of each peptide from the 3 strains immunized to yield maximum responses to sporozoite antigens are shown in Figure 5. As shown, proliferation in all 3 strains was strictly limited to the sporozoite antigens and could not be recalled with the CS peptides.

DISCUSSION

To enhance our understanding of the immune protection against the protozoan parasite *Plasmodium,* the rodent malaria species *P. berghei* was used as a model to evaluate the sporozoite-induced T cell specific repertoire directed against the sporozoite antigens. During the course of this study, we observed that the sporozoite-specific proliferative T cell repertoire was inducible in both lymph nodes and the spleen; however, proliferative responses of splenic lymphocytes varied not only according to the immunizing regimen, but also according to the murine strain. These fluctuations may indicate different mechanisms of immune protection in each murine strain examined. Furthermore, a very limited CS

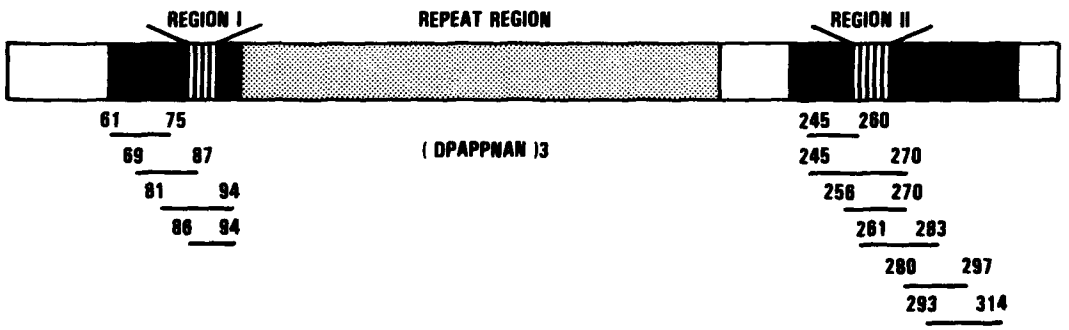

FIGURE 4. Model of the *P. berghei* CS protein. There is a long central repeat region consisting of the amino acid sequences DPAPPNAN and DPPPPNPN. Two short regions flanking the repeating sequence are highly conserved among different species of *Plasmodia* and are referred to as Region I and Region II.

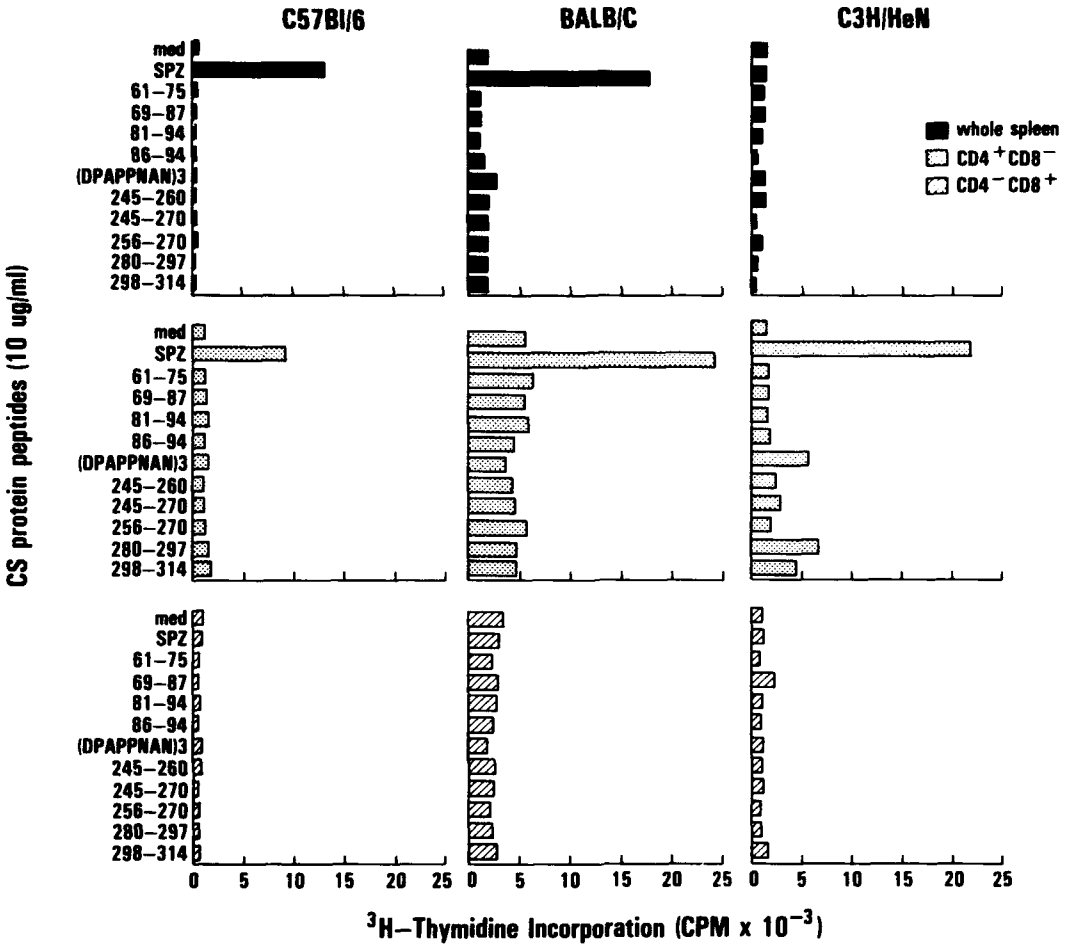

FIGURE 5. Reactivity of sporozoite-immune splenic lymphocytes to CS protein synthetic peptides. BALB/c, C57BL/6, and C3H/HeN mice were immunized iv with irradiated sporozoites using doses yielding optimal anti-sporozoite antigen responses, as described for Fig. 2. Sporozoite-specific spleen cells, enriched $CD4^+$ T cells, or enriched $CD8^+$ T cells were cultured in the presence of *P. berghei* CS protein synthetic peptides at concentrations of 0.01–10 μg/ml. Data represent responses elicited with 10 μg/ml of each peptide and are expressed as mean responses of triplicate cultures in cpm of ^{3}H-TdR incorporation during the last 16 hr of culture.

protein-specific T cell repertoire is induced upon immunizing with sporozoites, implying either restricted utilization of the CS protein-specific T cell repertoire or quite limited CS protein processing.

Induction of sporozoite-specific T cells in the lymph nodes and the spleen

Analysis of the sporozoite antigen-specific T cell repertoire was investigated in sporozoite antigen-induced splenic cultures. Earlier reports have suggested that the sporozoite is mitogenic and therefore could not be used as an in vitro antigen.[24] The data presented here demonstrate that sporozoite-primed splenic and lymph node lymphocytes respond in vitro specifically and in a dose-dependent fashion to sporozoite antigens treated by freezing/thawing and irradiated 30,000 rads.

The sporozoite-specific proliferative response was not an expected result since sporozoite immunizations were done iv, which typically favors the induction of T suppressor cells.[25] Another unexpected observation was that the induction of sporozoite-specific T cells varied according to the murine strain, implicating the involvement of an entire host of factors influencing the im-

TABLE 2

Proliferative activity of CD4⁺ T cells and protective immunity against sporozoite challenge in C3H/HeN mice

Strain	SPZ/well	Spleen cells cpm × 10^{-3}	CD4⁺ T cells	No. protected
C3H/HeN				
Exp. 1	0	0.48	0.82	
	300	0.68	1.34	
	1,000	0.82	1.21	
	3,000	1.66	9.51	5/6
Exp. 2	0	1.63	1.57	
	300	7.33	5.8	
	1,000	12.98	11.9	
	3,000	13.32	21.88	5/5
Exp. 3	0	2.32	1.66	
	300	2.4	1.27	
	1,000	2.17	1.46	
	3,000	1.06	1.18	0/4
Exp. 4	0	2.68	6.14	
	300	2.81	11.08	
	1,000	2.73	14.84	
	3,000	1.99	50.57	5/5
A/J				
Exp. 1	0	1.95	1.49	
	300	1.45	1.24	
	1,000	6.16	0.88	
	3,000	1.27	2.26	5/5

Data represent mean incorporation of ^{3}H-TdR from triplicate cultures ± 15% SD. Immunized mice were challenged with 10,000 live sporozoites and screened for parasitemia 7–14 days post-challenge. Naive mice were challenged for infectivity for each experiment. All animals displayed parasitemia within 7 days following challenge.

mune status, e.g., the background genes, MHC genes, the minor lymphocyte stimulatory determinants,[26] or IL-2 receptor (IL-2R) density.[27] Likewise, sporozoite-induced antibody responses specific for the CS repeat region showed variation among these strains, although they do not reflect the fluctuations observed in the T cell proliferative responses (data not shown). Furthermore, the apparent inverse relationship between the onset of immune protection and proliferative responses is not well understood; functionally unique T cell types might have been generated in each strain following each immunization regimen. Although it is not entirely clear why the onset of immune protection is accompanied by depressed lymphoblastic responses, it has been suggested that the decrease in the IL-2R density on lymphocytes derived from mice infected with *Leishmania* is partly responsible for the proliferative nonresponsiveness;[28] however, other mechanisms of immunosuppression could be involved in anti-sporozoite responses.

Since priming with sporozoite-CFA induced proliferative responses in lymph node cells of all 3 strains, the strain-associated variation seen in the spleens cannot be attributed to a general low/high immune potential of each strain. Instead, iv priming with irradiated sporozoites may have induced some regulatory T cells generally not present in the lymph nodes upon peripheral immunization. Moreover, it has been well established that the induction of regulatory T cells is influenced by the antigenic dose, the route of antigen administration, as well as the form of the priming antigen.[25] Alternatively, iv immunization with irradiated sporozoites may have indirectly affected lymphocyte reactivity, for example, by triggering a release of prostaglandins that are known to induce nonspecific suppression of the the immune system.[29] It can, therefore, be concluded that the sporozoite antigen-specific T cell repertoire is readily available. The fluctuations observed in the splenic lymphocyte cultures may have been contributed by the parasite-induced pathology altering the general immune functioning. Furthermore, since it has been shown that only irradiated sporozoites administered iv induce protective immunity, the host-parasite relationship may result in regulatory interactions not found upon sporozoite-CFA priming.

What accounts for the shift in reactivities to sporozoites?

Depletions of CD8⁺ T cells from the BALB/c and C57BL/6 splenocytes did not reverse the nonproliferative status and did not significantly enhance CD4⁺ T cell reactivity, suggesting that nonresponsiveness is not regulated by CD8⁺ T cells. Either sporozoite priming does not induce regulatory CD8⁺ T cells, or their activity is targeted onto cells other than the proliferative CD4⁺ T cells. Alternatively, proliferative unresponsiveness seen in BALB/c mice upon priming could be caused by the in vivo activation of CD8⁺ T cells, thus preventing an early induction of CD4⁺ T cells. Suppressed proliferation in C57BL/6 mice following full immunization could reflect regulator activity of CD8⁺ T cells. Regulatory interactions of sporozoite-induced T cells remain unknown, but it has been demonstrated in the β-galactosidase system that CD8⁺ T cells inactivate CD4⁺ T cells,[4] and that priming with a peptide bearing suppressor determinant prevents the induction of helper T cells.[30]

In contrast, nonresponsiveness of C3H/HeN

splenic lymphocytes to sporozoite antigens was usually reversed by the removal of $CD8^+$ T cells. Interestingly, in the instances when the $CD4^+$ T cells proliferated to sporozoites, the mice were protected. It appears, therefore, that the $CD4^+$ T cells proliferative activity associated with immune protection may be unique to the C3H/HeN mouse strain. This point is of particular interest since C3H/HeN and A/J mice share all MHC loci, except the D locus, coding for class I antigen. Although we can only speculate about the cellular mechanisms involved in sporozoite-induced immune protection, these observations strongly suggest multifactorial mechanisms involving not only unique T cell specificities, but also diverse T cell functions. For example, phenotypically identical $CD8^+$ T cells suppress proliferative $CD4^+$ T cells in the C3H/HeN strain, yet they might function as cytolytic T lymphocytes in BALB/c and C57BL/6 mice. The induction of presumably diverse functional T cells in different mouse strains may be triggered by a particular strain of *P. berghei* found within the uncloned NK65 *P. berghei* sporozoites. An important degree of variability in the gene coding for the CS protein has been noted within different strains of many *Plasmodia* species.[31,32] Supported by the apparent differences in the sporozoite-specific T cell sets, these findings underscore the uniqueness of *P. berghei* sporozoite-specific immune responses generated in different murine strains.

It can also be postulated that the lack of proliferation reflects the induction of non-proliferating $CD4^+$ T helper cells and that they define the protective status in some mouse strains. If only $CD4^+$ T helper cells were induced in BALB/c mice, they typically would not be identified in the proliferative assay system. In the C57BL strain, on the contrary, a priming dose might have preferentially induced the proliferative $CD4^+$ T cells, which are known to produce the IL-2 and gamma interferon required for the induction of functional T cell sets and the activation of macrophages, respectively.[33] In this case, the $CD4^+$ T helper cells would be induced only after a secondary or a tertiary immunization, at which time the proliferating $CD4^+$ T cells would become quiescent.

CS protein T cell specificity repertoire is highly restricted

The inducibility of sporozoite-specific T cells implied the presence of a T cell repertoire with specificities directed to epitopes on the CS protein. Surprisingly, peptides were ineffective in recalling T cell proliferative responses. Two-fold responses appeared sporadically to various peptides; however, the significance of these activities remains unclear, as stimulation was inconsistent and insensitive to antigen concentrations. Anomalous peptide responses could be due to minor T cell populations arising after sporozoite priming and could also reflect shifts in T cell specificities caused by variations in the sporozoite preparations.

Although B cell epitopes have been found within the repeat region of the CS surface protein, it is possible that the immunodominant T cell epitope is localized elsewhere on the sporozoites. This may account for the lack of CS protein peptide reactivity. Results from the hepatitis B system have shown that while the B cell epitopes are found on the surface of the antigen, the T cell epitopes map within the nuclear core protein, with intermolecular collaboration leading to an antibody response.[34]

Alternative reasons for the absence of peptide reactivity may be that determinants not represented by the available CS protein peptides are responsible for the activation of the sporozoite-specific T cells, or that processed antigenic fragments containing the dominant T cell epitopes assume a secondary structure not preserved within the short peptide residues. It could be postulated that CS protein peptides do not associate with MHC molecules, a necessary step for T cell triggering.

Restrictions imposed during processing and presentation of the authentic CS protein may also account for the lack of peptide reactivity. Since macrophages, dendritic cells, B cells, and other cells can function as antigen presenting cells (APC), antigen uptake and metabolic fragmentation may proceed quite differently in each case owing to the inherent differences in the way antigens interact with each cell type. For example, B cells usually bind antigen specifically via the Ig receptor,[35] whereas macrophages phagocytize antigens.[36] Consequently, antigens processed by B cells may yield determinants distinct from those displayed by macrophages.

It has been observed that sporozoites seldom interact with macrophages in vitro, and in some cases the sporozoite has been shown to penetrate, escape, and re-penetrate the macrophage, a scenario analogous to a needle and thread (J. P.

Vanderburg and others, New York University School of Medicine, New York, NY, personal communication). Often this interaction leads to the destruction of macrophages. If the same occurs in vivo, other cells would be expected to function as APC to initiate anti-sporozoite T cell responses. It remains unknown whether other types of APC are engaged or whether the sporozoite antigen is internalized and processed before presentation.

In summary, our observations underscore the findings that immune responses to sporozoite antigens are extremely variable among murine strains, implying multifactorial mechanisms in protective immunity. Furthermore, the lack of anti-CS protein peptide reactivity strongly suggests presence of antigens other than the CS protein to be involved in the anti-sporozoite response, thus providing an impetus for identification of additional antigens for vaccine development.

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